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Fibrinolysis, Inhibitors of Blood Coagulation, and Monocyte Derived Coagulant Activity in Acute Malaria

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Different parameters of fibrinolytic systems like t-PA, PAI, D-dimer, and inhibitors of blood coagulation, i.e., protein C (PC), protein S(PS), and antithrombin III (AT-III), have been studied in cases of acute malaria due to *Plasmodium falciparum* and *plasmodium vivax* infection, and these patients were followed up. It was observed that the plasma PAI-1 was very high in cases of *P. falciparum* malaria infection as compared to normal controls and *P. vivax* infection. The changes in complicated cases of *P. falciparum* were remarkable as compared to uncomplicated ones. The PC, PS, and AT-III levels were also low in *P. falciparum,* particularly so in complicated cases, and were normal in *P. vivax* infection. The factor VIII R:Ag levels were invariably high in acute malaria. On follow-up of some of these cases the values came back to normal after the antiparasite treatment. The monocyte procoagulant activity was found to be significantly higher in *P. falciparum* infection as compared to that of *P. vivax* infection. All these findings therefore contribute towards the production of a hypercoagulable state in *P. falciparum* infection and partly explain the complications of *P. falciparum* infection like cerebral malaria. Am. J. Hematol. 54:23–29, 1997

Key words: acute malaria; fibrinolysis; procoagulant activity; complications

INTRODUCTION

Malaria remains a major cause of morbidity and mortality in tropical and subtropical countries. With a resurgence of malaria in India, a large number of cases of cerebral malaria is encountered in various parts of India. Altered hemostasis associated with plasmodium falciparum infection has been reported in the past. Consumption of clotting factors and high levels of fibrinogen degradation product (FDP) were found in some studies [1–3]. However, other investigations have reported only isolated thrombocytopenia without any alteration in the coagulation system [4,5]. The complications of malaria include cerebral involvement, renal failure, pulmonary edema, and hepatic dysfunction, etc. Many of these complications are believed at least in part to be related to the hypercoagulable state in this disease. Our previous studies [6,7] showed alteration of the platelet function and increased procoagulant activity of parasitised RBC in the case of *P. falciparum* infection. These factors are believed to be responsible for the hypercoagulable state in acute malaria cases particularly due to *P. falciparam* infection. Therefore, we attempted to study the different parameters of the fibrinolytic system as well as the inhibitors of blood coagulation. This may help us to understand the pathogenesis of various complications associated with malaria and thereby develop a means of prevention or appropriate therapy for such complications.

PATIENTS AND METHODS

Patients

This study includes 100 adult patients, both male and female, presenting at the New Civil Hospital, Surat, India fulfilling the following criteria.

1. Febrile illness of any duration.
2. Presence of malaria parasite in peripheral blood smear examination.

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3. No evidence of any other infection.
4. No past history or family history of bleeding.
5. Exclusion of other causes of unconsciousness and coma in suspected cases of cerebral malaria by appropriate investigations including CT scan of the head.

The level of unconsciousness was assessed by the Glasgow coma scale. Complete physical examination was done on admission with particular reference to pallor, icterus, petechiae, purpura, ecchymosis, hepatosplenomegaly, and anaemia, etc.

Laboratory Investigations

Malaria parasite demonstration. The thick and thin peripheral blood smears were prepared immediately on admission, and after staining with Romanowsky stain (Giemsa) examined under the oil immersion lens. The parasite density was recorded as follows following the criteria of Werndosfer and McGregor [8].

+	1–10 parasites per 100 oil immersion fields.
++	> 10 parasites per 100 oil immersion fields.
+++	1–10 per 1 oil immersion fields.
++++	> 10 parasites per 1 oil immersion fields.

Venous blood was collected in 3.8% sodium citrate with 9:1 proportion of blood to anticoagulant and transported over ice and immediately centrifuged at 4°C for 10 min (1,500 g) to prepare platelet poor plasma (ppp).

tPA and Protein C were estimated both by ELISA and chromogenic assay. Plasminogen activator inhibitor (PAI-I) and total protein S were estimated by ELISA, anti thrombin III was estimated by chromogenic assay, and D-Dimer was assessed semiquantitatively with a latex agglutination test. All tests were done with appropriate positive and negative controls. The kits for these tests were obtained from Diagnostica Stago, Asnieres, France. Detailed tests of fibrinolysis, natural inhibitors of blood coagulation, were done on 41 patients (31 patients of *P. falciparum* and 10 patients of *P. vivax*).

Bleeding time was done using modified Ivy's technique. Routine investigations included CBC, serum biochemistry chest X-ray and screening coagulation tests in all patients [9].

Factor VIII R:Ag. It was estimated by Laurell rocket technique utilising antiserum to human factor VIIIR:Ag (Dakopatts, Glostrup, Denmark) in 41 cases (31 *P. falciparum* plus 10 *P. vivax*).

Procoagulant Activity of Peripheral Blood Monocytes

For the study of procoagulant activity of the monocytes, citrated blood was separated over lymphoprep (Nyyegard, Oslo, Norway). The mononuclear cells were washed twice with PBS (pH 7.2) and a count was done by cell counter (Erma PC608, Tokyo, Japan). The monocytes were separated from the lymphocytes by adhesion to plastic petric dishes for a period of 30 min. The cells in the supernatants contained the lymphocytes, which were removed. Monocytes (1×10^5) were suspended in 1 ml of MEM media and incubated at 37°C. The supernatants were checked for the procoagulant activity by plasma recalcification time (PRT) utilising normal pooled plasma. The PRT with media served as the control. The shortening of the PRT value indicated the procoagulant activity. The plasma recalcification time (PRT) of normal pooled plasma (pool of 10 samples) obtained on each day was taken as 100% in the calculation of the effect of adding supernatant from the peripheral blood monocyte short-term culture; this excluded daily variation of normal PRT. The fall in PRT after the addition of the culture supernatant was expressed as a percentage of the PRT of the pooled normal plasma obtained that day, i.e.,

$$\left(1 - \frac{\text{PRT after additiona of culture supernatant}}{\text{PRT of pooled normal plasma}}\right) \times 100$$

The greater the percentage the stronger the procoagulant activity [7,10].

The follow-up study was done in 17 patients after 7–10 days of treatment.

Statistical Analysis

Statistical analysis was done by non-parametric tests, i.e., the Wilcoxon paired difference tests and the Mann and Whitney U test using SPSS software package [11]. Correlations between different parameters were calculated by the Spearman rank test. Any P value of 0.05 or lower was considered to be significant.

RESULTS

Out of a total of 100 cases of acute malaria studied 24 had *P. vivax* and 76 had *P. falciparum* infection. In the group of *P. falciparum*, 39 had cerebral involvement. The mean age in *P. falciparum* infection was found to be 31.63 ± 13.55 whereas the mean age in the *P. vivax* group was 28.37 ± 7.4. There were 51 males and 25 females in the *P. falciparum* infection group and the *P. vivax* group had 16 males and 8 females.

All patients had a history of fever with chills and rigor of varying duration (mean 6.56 days). The other clinical features were vomiting in 54% of cases, headache in 31% of cases, and pallor was detected in 80% of patients. Splenomegaly was present in 46% and hepatomegaly in 26% of cases. In the case of *P. falciparum* infection, the cerebral manifestations were altered consciousness,

convulsions, and paresis. History of bleeding was present in only 4 cases, out of which 3 had epistaxis, 2 had bleeding gums, and 1 had bloody diarrhoea.

Chloroquine was given to 68 patients. Other drugs used were quinine in 40 patients, sulphamethaxazole plus pyrimethamine in 12 patients, tetracycline in 23 patients, primaquin in 3 patients, and mefloquine in 1 patient. The cases of cerebral malaria are all treated by quinine intravenously followed by oral quinine. One case resistant to chloroquin and sulphamethaxozole was treated with mefloquine. Out of the 100 patients, 89 were cured, 9 expired (all were cases of cerebral malaria), and 2 left against medical advice.

Screening Coagulation Tests

Prolonged BT ($>$ 7 min) was seen in 4 cases (normal 1–3 min). All 4 had *P. falciparum* infection. Three out of these 4 were cases of cerebral malaria. On follow-up, the BT became normal in 2 patients and the other 2 cases expired.

Only in 4 cases of cerebral malaria, was PT significantly prolonged ($>$ 16 secs). Eight patients had prolonged APTT ($>$ 45 sec). All these cases were of *P. falciparum* infection and 5 of them had cerebral malaria.

Platelet Count

In 39% of cases of *P. falciparum* infection thrombocytopenia (platelet count $< 150 \times 10^9$/l) was present, whereas only 29% of *P. vivax* infection had thrombocytopenia. The thrombocytopenia was more severe in falciparum infection (mean $165 \pm 78 \times 10^9$/l) as compared to *P. vivax* (mean $170 \pm 47 \times 10^9$/l). The thrombocytopenia of complicated *P. falciparum* infection was severe as compared to uncomplicated cases. Further the number of cases with thrombocytopenia is also more in complicated *P. falciparum* infection, but the difference was not statistically significant. There was no significant correlation between platelet count and parasite density. These platelet counts were taken at presentation, hence they were not confounded by effects of therapy.

Fibrinolytic Parameters

Tissue plasminogen activator (tPA). When the antigenic determination was done by ELISA assay in 39 cases (31 PF + 8 PV) it was found that in *P. falciparum* infection the values were lower as compared to *P. vivax* infection, although the difference was not statistically significant (Fig. 1). However, there was a reduction in the level of tPA in both the groups as compared to normal.

When tPA was estimated by chromogenic assay there was a significant reduction in the plasma level in malaria cases as compard to the normal control. This reduction was more in falciparum malaria as compared to *P. vivax.*

PAI-1 level. The values shown in Figure 1 indicate that plasma PAI-1 was elevated significantly as compared to normal in 60% of cases of malaria. This is more so in the cases of *P. falciparum* infection. It is interesting to note that PAI-1 level in plasma increased with an increase in the duration of fever.

D-Dimer. No D-Dimer could be demonstrated in 10 cases of *P. vivax* infection. *P. falciparum* infection showed a very strong reaction in two cases and in the other two it was weakly positive out of 31 cases tested. After 10 days of follow-up in two cases, the D-Dimer was found to be negative. The other parameters like protein S, protein C, and AT-III level were also significantly low in these cases. The screening coagulation tests like PT and APTT were prolonged and together with thrombocytopenia, which was present in these cases, established acute DIC. Both cases had cerebral involvement and features of pulmonary edema.

Plasma Factor VIII R:Ag

The values were highest in cases of complicated *P. falciparum* infection as compared to uncomplicated cases and cases with *P. vivax* infection (Fig. 2).

Inhibitors of Blood Coagulation

The values are shown in Figure 3. The important findings were the low values of Protein C in 40% of cases of *P. falciparum* infection and more so in cerebral malaria cases. The protein S level was also low in 19% of cases of *P. falciparum* and only in one case (10%) of *P. vivax* infection. Furthermore, the value was also on the borderline in this case. There was no lowering of the AT-III level in *P. vivax* infection, whereas 30% of cases of *P. falciparum* showed significant lowering.

Monocyte Procoagulant Activity The values are shown in Table I. There is a significant difference ($P < 0.001$) in the procoagulant activity of the culture supernatants of monocytes from *P. falciparum* as compared to *P. vivax* infection. The monocytes were probably stimulated in an in vivo situation and the maximum release of this activity was found after 2 hr of short-term culture.

DISCUSSION

The present study clearly demonstrates a significant alteration in the hemostasis in general and fibrinolysis in particular in acute malaria due to *P. falciparum* infection. This alteration was tilted towards the hypercoagulable state which tends to revert back to normal after antimalarial treatment. This process was more pronounced in complicated cases, i.e., with cerebral, renal, or pulmonary involvement. The follow-up study showed that even after 10 days of treatment some of the parameters did not reach the normal values. Bleeding was present only in 4 cases;

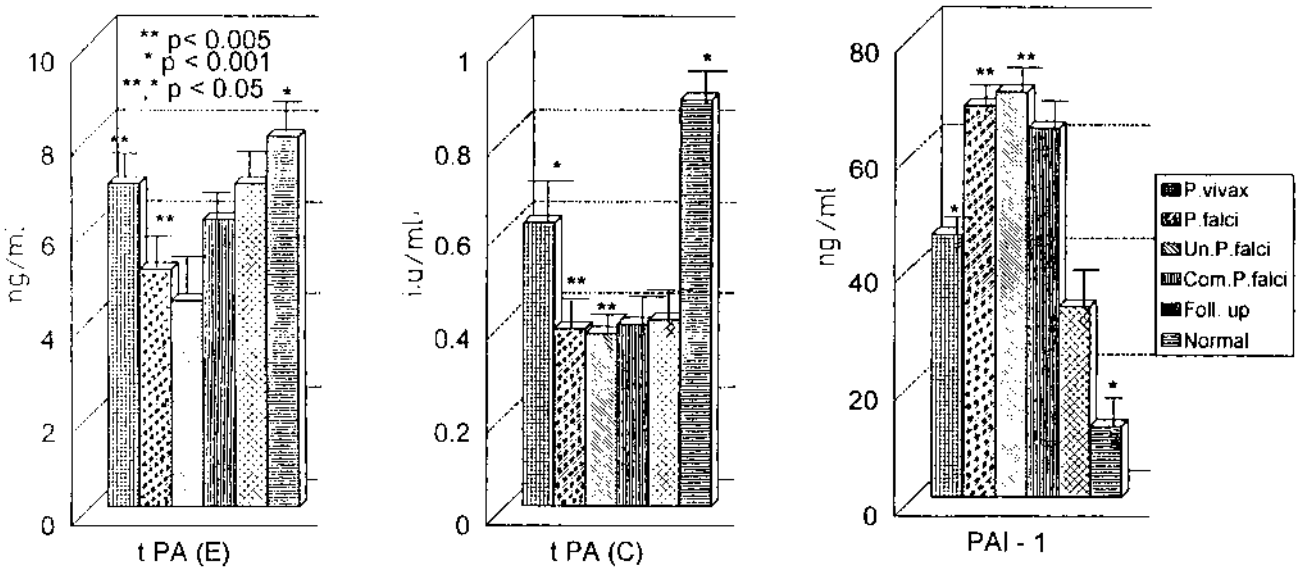

Fig. 1. Fibrinolytic parameters in various subgroups of malaria.

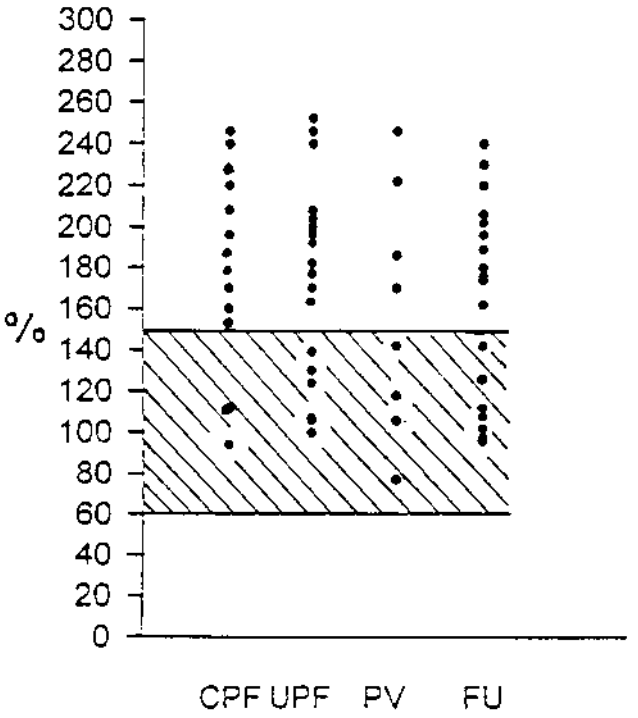

Fig. 2. Plasma VWF levels in malaria. CPF, complicated *plasmodium falciparum;* UPF, uncomplicated *plasmodium falciparum;* PV, *plasmodium vivax;* FU, follow up.

out of these, 3 had epistaxis, 2 had bleeding gums, and 1 had bloody diarrhoea. Bleeding in severe malaria may result from several pathological processes: thrombocytopenia, reduced clotting factor synthesis, and consumptive coagulopathy. Activation of the coagulation cascade occurs in uncomplicated malaria but this is mild in degree and reverts to normal as the patient becomes afebrile and aparasitemic [12].

In severe cases of *P. falciparum* infection it proceeds to a hypercoagulable state ultimately leading to disseminated intravascular coagulation. The mechanisms underlying the activation of the coagulation cascade in severe malaria are unclear. However, one of the mechanism reported is the activation of the intrinsic pathway of the clotting cascade in *P. falciparum* infection [13]. Activation of the intrinsic pathway is often associated with activation of the fibrinolytic system. Previous studies in severe malaria have shown consistently elevated concentrations of fibrin degradation products, usually with increased levels of fibrinogen, suggesting increased fibrinogen turnover [1,14,15]. However, there is a paucity of reports regarding the study of different components of fibrinolytic system. Furthermore, only 2 studies are available in which only protein C level or AT-III level, respectively, in malarial infection [16,17] has been studied. Hence, determination of the functional as well as the antigenic level of PAI and t-PA is of evident clinical importance. Simultaneous determination or other inhibitors of blood coagulation like protein C and protein S and AT III will throw light on the pathophysiology of the hypercoagulable state seen in *P. falciparum* infection. The current study was designed to investigate simultaneously the different parameters of blood coagulation, fibrinolysis, and natural inhibitors of blood coagulation in acute malarial infection. Such a detailed study is scarce in the literature. Moreover, the present study explores the natural inhibitors of blood coagulation and fibrinolytic system in acute malaria and presents evidence of a significant alteration in the fibrinolytic system. A low AT-III level was seen in cases of *P. falciparum* infection as opposed to the previous finding of a normal AT-III level in *P.*

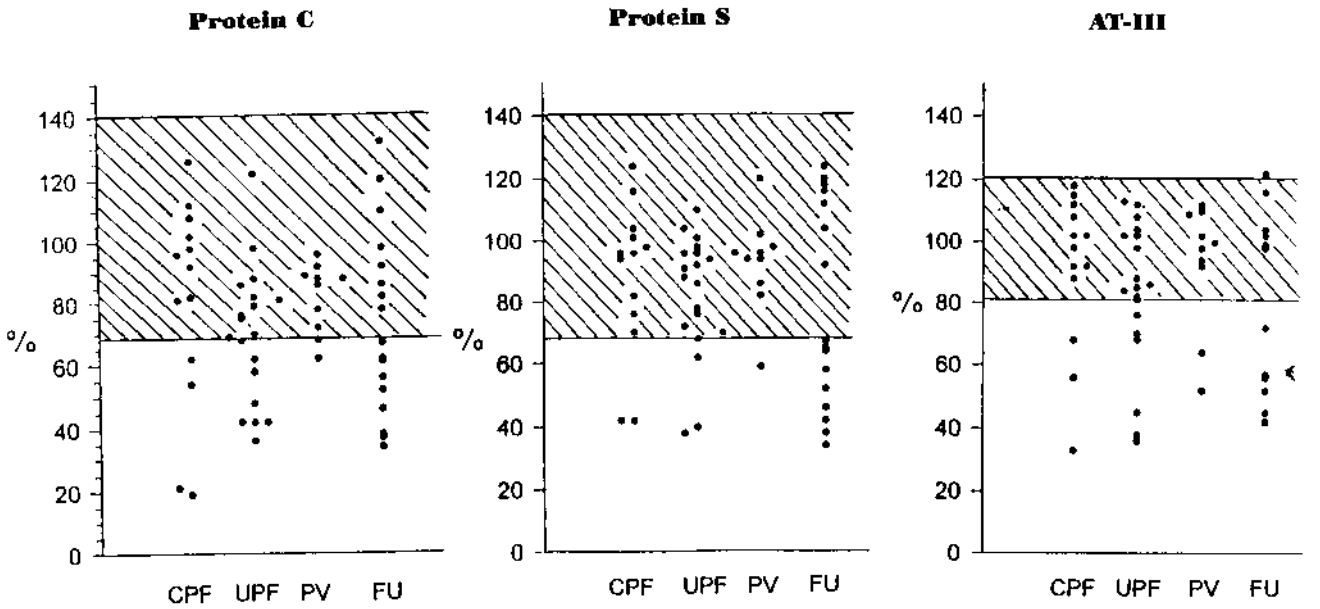

Fig. 3. Inhibitors to blood coagulation factors in malaria. For abbreviation definitions see Figure 2.

TABLE I. Monocyte Procoagulant Activity in Malaria*

	% of Shortening of PRT (hr)			
	2	4	8	14
P. vivax (n = 12)	22.16 ± 7.56	16.8 ± 7.94	12.5 ± 7.60	8.08 ± 7.21
Uncomplicated *P. falciparum* infection (UPF) (n = 5)	35.2 ± 4.35	30 ± 5.29	23.6 ± 6.37	10.6 ± 4.27
Complicated *P. falciparum* infection (CPF) (n = 7)	55.28 ± 4.26	50.28 ± 5.00	42.85 ± 3.52	29.85 ± 4.01
Healthy control (n = 21)	8 ± 1.63	6.5 ± 3.87	5 ± 1.73	4 ± 1.15

*P. *vivax* vs. UPF, $P < 0.001$; *P. vivax* vs. control, $P < 0.001$; *P. vivax* vs. CPF, $P < 0.001$.

falciparum infection in spite of high parasitemia [17]. However, our finding of low AT-III levels in *P. falciparum* infection is in agreement with another earlier report [12].

Reduction in protein C level correlated ($r = 0.44$, $P < 0.01$) well with the coma scale in cerebral malaria cases and returned back to normal after 2 weeks. This confirms the observation of a previous study [16]. There is also sufficient evidence of the generation of thrombin in malaria. Therefore, the reduction in the levels of protein C, protein S, and AT-III strongly suggests the consumption of these clotting factors due to microvascular thrombosis rather than due to reduced synthesis in the liver. On follow-up, reduced levels of protein S, protein C, and AT-III persisted till day 10. A study conducted in Thailand showed AT-III levels reached 75% of normal value by the 7–10th day of infection [12].

Our observation of an increased PAI-1 antigen level observed in patients with acute malaria is probably due to parasitemia. Two distinct explanations may be offered for the rapid increase of PAI-1: (1) TNF-alpha produced by the leucocytes inducing neosynthesis and release from the endothelial cells, and (2) release of PAI-1 from the platelets. Previous studies have shown that platelets are a major source of PAI-1 in human blood [18–21] and approximately five to six times more PAI-1 is found in platelets than in plasma [22]. Although the tissue plasminogen activator (tPA) estimation was low by both methods, there was not a good correlation between the functional estimation and antigenic determination ($r = 0.23$, $P > 0.05$). This discrepancy noted between tPA activity and tPA antigen may be partly due to the measurement of complexes of PAI-1 with tPA in ELISA assay. Furthermore, part of tPA may be in the "inactive" latent form not detected by functional assay. There was a very good

correlation between elevated PAI-1 levels with reduction in tPA levels in acute malaria, with a trend towards normalisation of these levels on follow-up. This implies ineffective fibrinolysis and a tilting of hemostatic balance towards the hypercoagulable state. TNFalpha reduces the secretion of tPA and increases the secretion of PAI-1. The elevation of PAI-1 strongly correlated with a decrease in the coma scale in cerebral malaria patients and also correlated inversely with a reduction in platelet count, and protein C, protein S, and AT III levels. TNF-alpha plays an important role in the pathogenesis of cerebral malaria. The cerebral complications of murine malaria can be prevented by administration of anti-TNF antibodies prior to infection [23]. It has also been reported that parasitised RBC and malarial proteins interact with macrophages in vitro [24] and in vivo [25] to induce production of TNF alpha. In vitro, TNF-alpha induces procoagulant activity synthesis in cultured endothelial cells [26,27]. The increase in factor VIII R:Ag was found in the majority of cases of falciparum malaria and in some cases of *P. vivax* infection. The increased level correlated well with parasite density ($r = 0.46$, $P < 0.01$). Similar findings have been reported earlier [17]. About 90% of circulating vWF is synthesized and secreted by the endothelial cells.

The increased level of vWF in malaria indicated endothelial damage by the parasitised RBC/parasite. The high level of factor VIII R:Ag (vWF) might be contributing towards the production of the hypercoagulable state in acute malaria. The mechanism involved may be release of high molecular weight vWF from damaged endothelial cells. These molecules can trigger platelet aggregation and contribute to the hypercoagulable state.

D-dimer was positive only in four cases of complicated *P. falciparum* malaria showing that DIC is not a common complication in these cases. Our findings are in line with that of Pukrittayakamee et al. [12].

The release of procoagulant activity (tissue factor) from the monocytes as a result of stimulation by the parasite may compound the pathogenesis of severe *P. falciparum* malaria (Table I).

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